Modulation of Metastasis Phenotypes of Non-Small Cell Lung Cancer Cells by 17-Allylamino 17-Demethoxy Geldanamycin

Dao M. Nguyen, MD, Sudhen Desai, MD, Aaron Chen, MS, Todd S. Weiser, MD, and David S. Schrump, MD

Thoracic Oncology Section, Surgery Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Background. Cancer cells that overexpress c-erbB oncogenes exhibit resistance to chemotherapy, enhanced tumorigenicity, as well as increased propensity for metastasis. The aim of this study was to investigate if depletion of erbB-1/EGFR and erbB-2/HER2neu oncogene products by 17-allylamino 17-demethoxy Geldanamycin (17AAGA) could diminish the metastatic potential of non-small cell lung cancer (NSCLC) cells that express varying levels of the erbB1/erbB2 oncogenes.

Methods. NSCLC cell lines (H460, H358, H322, or H661) were assayed for expression of erbB1 and erbB2, the cell adhesion molecule E-cadherin, secretion of the matrix metalloproteinase 9 (MMP-9), and vascular endothelial cell growth factor (VEGF), as well as their ability to invade Matrigel after 48-hour exposure to 17AAGA.

Results. 17AAGA significantly depleted erbB1 or erbB2 levels in NSCLC cells expressing high levels of these proteins, and effectively inhibited their growth with IC_{50} values ranging from 50 to 90 nmol/L. Moreover, drug treatment enhanced E-cadherin expression in H322 and H358 cells, and inhibited secretion of MMP-9 and VEGF secretion by tumor cells. 17AAGA diminished hypoxia-induced upregulation of VEGF expression as well as growth factor-mediated augmentation of MMP-9 secretion, and profoundly inhibited the ability of H322 and H358 cells to migrate through Matrigel in response to chemoattractants.

Conclusions. In addition to its known antiproliferative and chemosensitization effects, 17AAGA inhibits the metastatic phenotype of lung cancer cells. 17AAGA may be a novel pharmacologic agent for specific molecular intervention in lung cancer patients.

(Ann Thorac Surg 2000;70:1853–60)

Patients with non-small cell lung cancer (NSCLC) frequently present with either locally advanced (stage IIIA/B) or systemic disease (stage IV). The overall prognosis for these individuals is very poor, with the median survival less than 9 to 12 months despite an aggressive combination of chemo and radiotherapy. More importantly, the main mode of treatment failure after curative-intent therapy for early-stage NSCLC is systemic metastasis [1]. Acknowledging the fact that mature data of phase II/III clinical trials addressing the values of adjuvant chemotherapy for completely resected early-stage NSCLC are still forthcoming, there is no evidence that adjuvant therapy has any impact on the outcome of surgically treated NSCLC [2]. Micrometastases derived from lung cancers may have low proliferative activity, rendering them insensitive to standard cytotoxic agents. Hence, there is an urgent need for novel antineoplastic agents that exhibit strong antiproliferative effects as well as a capacity to inhibit the metastatic potential of tumor cells.

Solid tumor metastases arise by a multistep process regulated by complex interactions between tumor cells and adjacent stromal tissues [3]. In association with altered expression of adhesion molecules such as E-cadherin, cells detach from the main tumor mass and migrate through the extracellular matrix, degraded by tumor-derived matrix metalloproteinases (MMPs). After dissemination, cells exit from the vasculature and establish metastatic deposits, facilitated by the expression of integrin or hyaluronate (CD44) receptors, and the secretion of angiogenesis factors such as vascular endothelial cell growth factor (VEGF).

Experimental and clinical data have indicated that overexpression of erbB1 and erbB2 protooncogenes encoding the epidermal growth factor receptor (EGFR) and the orphan receptor (HER2/neu), respectively, correlates with locally advanced disease, distant metastases, and diminished survival in patients with breast, lung, esophageal, and ovarian carcinomas [4–7]. Tumor cells overexpressing these protooncogenes exhibit one or more phenotypes correlating with enhanced metastatic potential

Presented at the Thirty-sixth Annual Meeting of The Society of Thoracic Surgeons, Fort Lauderdale, FL, Jan 31–Feb 2, 2000.

Address reprint requests to Dr Nguyen, Thoracic Oncology Section, Surgery Branch, Division of Clinical Sciences, National Cancer Institute, Room 2B07, Building 10, 10 Center Dr, Bethesda, MD 20892; e-mail: dao_nguyen@nih.gov.

Published by Elsevier Science Inc

0003-4975/00/$20.00
PII S0003-4975(00)01810-5

including downregulation of E-cadherin expression, increased secretion of MMP-9 and VEGF, and accelerated invasion through extracellular matrix [8–10]. Therapeutic strategies that specifically target erbB should inhibit mitogenic signaling via erbB1 and erbB2 pathways and may reduce the proliferation and metastatic potential of cancer cells; indeed, treatment of cells overexpressing the erbB1 or erbB2 gene products with antagonistic monoclonal antibodies significantly inhibits their metastatic phenotype [10, 11].

The benzoquinone ansamycin antibiotic geldanamycin (GA) and its less toxic synthetic derivative, 17-allylamino 17-demethoxygeldanamycin (17AAGA), have been recently selected for clinical development at the National Cancer Institute based upon their activity against cell lines derived from a variety of human malignancies [12, 13]. The growth inhibitory effects of geldanamycin and 17AAGA appear related to their ability to inhibit the expression of several cellular oncoproteins, including erbB1 and erbB2 [13]. In the present study, we sought to evaluate if 17AAGA could modulate the metastatic potential of lung cancer cells overexpressing the erbB1 and erbB2 gene products. Herein, we demonstrate that treatment of NSCLC cells with 17AAGA results in significant reduction of erbB1 or erbB2 expression, profound inhibition of cell proliferation, upregulation of E-cadherin expression, and downregulation of VEGF and MMP-9 secretion, paralleling with diminished capacity to invade extracellular matrix in vitro.

Material and Methods

Cells and Reagents

The NSCLC cells H460, H358, H661, and H322 were purchased from ATCC (Manassas, VA), and were grown in RPMI-1640 medium supplemented with glutamine (1 mmol/L), penicillin (100 U/mL)/streptomycin (100 μg/mL), and 10% fetal bovine serum (FBS) (all purchased from Biofluids, Rockville, MD). Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics, Inc (Walkerville, MD) and maintained in bronchial epithelial cell basal media (Clonetics, Inc). 17AAGA was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and dissolved in dimethyl sulfoxide to yield a 100 μmol/L stock solution, which was stored at −70°C. All experiments using this compound were performed under subdued lighting conditions. Recombinant human epidermal growth factor (EGF) and heregulin-α (HRG) (purchased from R&D, Minneapolis, MN), and the anti-erbB1 and anti-erbB2 monoclonal antibodies (purchased from Calbiochem/Oncogene Research Products, Cambridge, MA), were constituted in phosphate-buffered saline (PBS), and stored at 4°C as recommended by the manufacturers. The E-cadherin antibody was obtained from ICN Biomedicals, Inc (Aurora, OH).

Immunofluorescent Staining and Flow Cytometric Analysis of erbB1, erbB2, and E-cadherin

Expression of erbB1, erbB2, and E-cadherin in NSCLC cell lines was quantitated by flow cytometry using a Becton-Dickinson (San Jose, CA) fluorescence-activated cell sorter (FACS). In brief, cells grown in either normal media with or without 17AAGA were harvested by trypsin/EDTA. To prevent proteolysis of surface adhesion molecules, cells harvested for E-cadherin staining were treated with 0.01% crystallized trypsin in the presence of 1 mmol/L of calcium, then washed in Ca/Mg-free PBS. Single-cell suspensions were incubated with either anti-erbB1, anti-erbB2, or anti-E-cadherin monoclonal antibodies for 60 minutes at room temperature. Cells were then washed with PBS, and incubated with fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody at room temperature for 60 minutes in the dark. An irrelevant mouse IgG isotype monoclonal antibody was used as a negative control for FACS analysis. A minimum of 10^4 cells were analyzed by FACS flow cytometry. The magnitude of surface expression of these proteins was indicated by the mean fluorescence intensity (MFI) of positively stained cells. The MFI of isotype IgG control samples was always less than 10.

In Situ Immunofluorescent Staining of E-cadherin

Cells were cultured in chamber slides (Nalge Nunc International Corp, Naperville, IL) until 80% confluent, washed with PBS, fixed with 1% paraformaldehyde for 10 minutes, and permeabilized with acetone for 5 minutes. Immunofluorescent staining for E-cadherin was performed similar to that described for flow cytometry. Slides were examined under the fluorescence microscope. Fluorescent photomicrographs were taken at 200× magnification.

MMP-9 Assay

Cells were grown to confluence in six-well plates, washed twice with PBS, and then incubated with 2 mL of fresh serum-free RPMI-1640 medium. In the 17AAGA treatment group, cells were replenished with media containing either 20 or 80 nmol/L of 17AAGA. After 48 hours of incubation, conditioned media were harvested and frozen at −70°C until assayed for MMP-9 expression using an enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem/Oncogene Research Products, Cambridge, MA). Cells from each well were collected, and total cellular protein was assayed by bicinchoninic acid (BCA) technique (Pierce, Rockford, IL). MMP-9 levels in the conditioned media were expressed as pg/mL/24 h/mg of total cellular protein.

VEGF Assay

Cells were grown to 80% confluence in 24-well plates, washed once with PBS, and replenished with 2 mL of fresh RPMI-1640 alone or containing either 50 μmol/L or 100 μmol/L Cobalt Chloride ($CoCl_2$) to simulate hypoxia [14]. In the 17AAGA treatment groups, appropriate aliquots of 100× 17AAGA stock were added to the culture

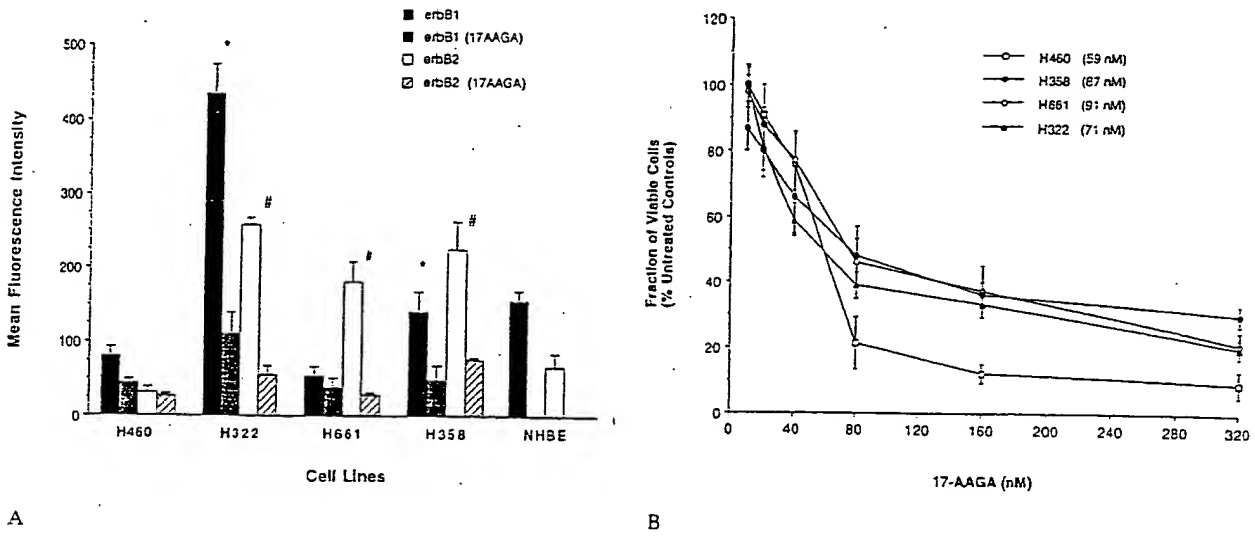

*Fig 1. (A) Flow cytometric analysis of basal expression and 17AAGA-mediated depletion of erbB1 and erbB2 surface receptors in 4 NSCLC cell lines and normal human epithelial cells (NHBE). Exposure of these cells to 80 nmol/L of 17AAGA for 48 hours resulted in significant reduction of surface expression of these two receptors. Data are expressed as means ± SD of three independent experiments (*p < 0.0001, #p < 0.001). (B) Dose-dependent growth inhibition of NSCLC cells in vitro by 17AAGA. Cells were seeded in 96-well plates and, after an overnight incubation, were continuously exposed to varying doses of 17AAGA for 96 hours. Viable cells were quantitated by MTT assay. IC_{50} values, estimated from these dose-response curves, are indicated in parentheses. Data are expressed as means ± SD of three independent experiments.*

media to yield a final concentration of 80 nmol/L. After 48-hour incubation, conditioned media were harvested and frozen at −70°C until assayed for VEGF. VEGF levels in the conditioned media were evaluated using an ELISA kit (R&D) and expressed as ng/mL/24 h/10^6 cells.

Matrigel Invasion Assay

The chemoinvasion assay was performed as previously described [15]. Briefly, polyvinylpyrrolidone-free polycarbonate filters (10-μm pore size; Neuroprobe, Gaithersburg, MD) coated with 500 μg/mL of Englebreth-Holm-Swarm murine sarcoma basement membrane extract (Matrigel; Sigma Chemical Co, St. Louis, MO) were placed in a modified Boyden chamber (Neuroprobe, Gaithersburg, MD). Matrigel was diluted to the desired final concentration using cold (4°C) serum-free RPMI-1640 media. Filters were then placed in 15 mL of 500-μg/mL Matrigel in 100 × 15-mm Petri dishes and rotated at 45 rpm overnight at 4°C. Filters were air dried under a sterile hood immediately before use. Cells (6 × 10^5/mL) were suspended in serum-free RPMI and added to the upper chamber (30,000 cells/well). The lower chamber contained serum-free conditioned media from cultures of NIH 3T3 cells as a chemoattractant. Chambers were incubated at 37°C in 5% CO_2 for 6 hours. At the end of the incubation, cells on the upper surface of the filter were aspirated off and the filters were fixed in methanol and stained with Diff-Quik II, a Wright-Geimsa stain (Baxter, McGaw Park, IL). Cells that had invaded the lower surface of the filter were counted by light microscopy and data were expressed as cells/5 high-power fields (HPF)/well.

Data Analysis

Data are expressed as means ± standard deviation (SD). Student's *t* test and one-way analysis of variance (with Bonferroni test for pairwise comparisons) were used for statistical analysis using Prism 2.0 software package from Graphpad Software, Inc (San Diego, CA).

Results

Constitutive Expression of erbB1 and erbB2 in 4 NSCLC Cells Lines

The NSCLC cell lines used in this study express different levels of erbB1 and erbB2 surface receptors as determined by flow cytometric analysis (Fig 1A). In comparison with the receptor levels expressed in confluent NHBE, H460 cells express low levels of both erbB1 and erbB2; H661 and H358 cells express high levels of erbB2, but not erbB1, whereas H322 cells overexpress both erbB1 and erbB2. Forty-eight-hour exposure of these cells to 80 nmol/L 17AAGA resulted in significant reduction of surface expression of erbB1 or erbB2 in cells exhibiting elevated levels of these receptors. In parallel with inhibition of erbB1 or erbB2 expression, continuous exposure of these cells to 17AAGA for 96 hours resulted in a dose-dependent inhibition of cell proliferation, with estimated IC_{50} (concentrations of drug that inhibit 50% of cell proliferation) values ranging from 60 to 90 nmol/L (Fig 1B).

Upregulation of E-Cadherin by 17AAGA

Expression of the calcium-dependent epithelial cell adhesion molecule E-cadherin was readily detectable by

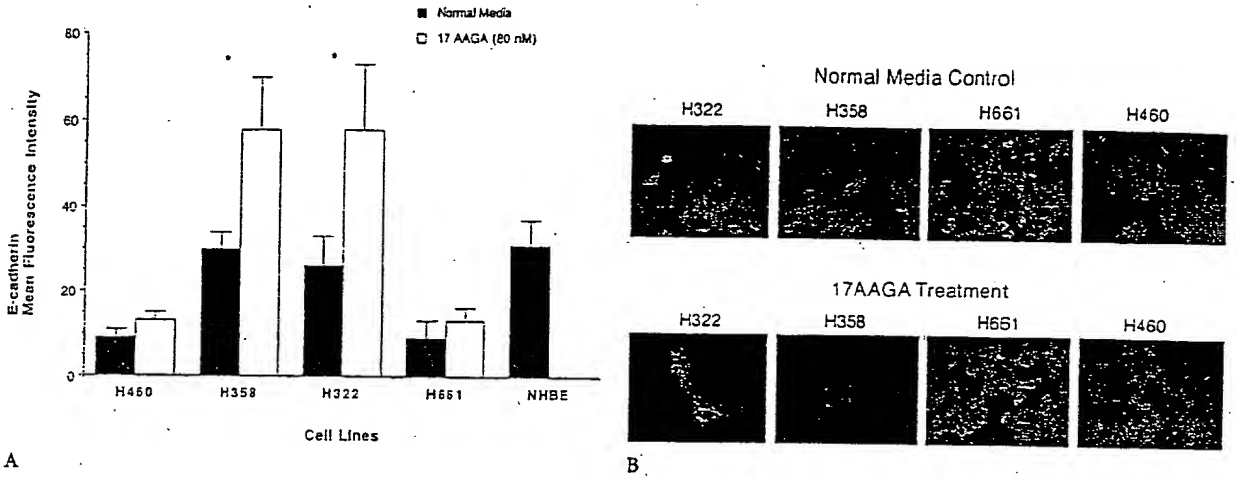

Fig 2. (A) Flow cytometric analysis of surface expression of E-cadherin on NSCLC cells under normal or 17AAGA condition. Similar levels of surface expression of E-cadherin were noted on H322, H358, and normal human bronchial epithelial cells (NHBE); E-cadherin was undetectable on H460 and H661 cells. Significant upregulation of E-cadherin expression was observed in H358 and H322 cells after 48-hour exposure to 17AAGA (80 nmol/L). Data are expressed as means ± SD of four independent experiments ($p < 0.01$). (B) Immunofluorescent analysis of lung cancer cells grown in normal media or media containing 17AAGA. After exposure to 17AAGA, H358 and H322 cells exhibit increased membrane localization of E-cadherin.*

flow cytometry in NHBE, H322, and H358 cells and not in H460 or H661 cells; results that were consistent with previously published data concerning E-cadherin expression in these cells [11]. The mean fluorescence intensity of E-cadherin in H322 and H358 cells increased 1.5- to 2-fold after treatment with 17AAGA (80 nmol/L for 48 hours) (Fig 2A). The percentages of positively stained cells also increased significantly after 17AAGA treatment in these cell lines (63% ± 13% and 58% ± 10% of 17AAGA-treated cells vs 47% ± 7% and 38% ± 8% of untreated H358 and H322 cells; $p = 0.05$ and $p = 0.02$, respectively). No upregulation of this adhesion molecule was noted in H460 and H661 cells. Although diffuse cytoplasmic staining for E-cadherin was noted both in control and 17AAGA-treated cells, intense fluorescence indicating localization of E-cadherin to the cell membrane was only observed in treated H358 and H322 cells. No such membrane localization was noted in H460 or H661 cells after 17AAGA treatment (Fig 2B).

Downregulation of MMP-9 Secretion

Continuous exposure of NSCLC cells to either 20 or 80 nmol/L of 17AAGA significantly inhibited MMP-9 secretion in a dose-dependent manner, with an overall reduction of up to 50% relative to baseline control levels (Fig 3A). Moreover, incubation of H358 and H322 cells, which express high levels of both erbB1 and erbB2 receptors with agonistic recombinant human EGF or heregulin-α, augmented secretion of MMP-9 by 35% to 50% relative to baseline. 17AAGA treatment completely blocked ligand-mediated upregulation of MMP-9 production in these two cell lines (Fig 3B).

Suppression of VEGF Secretion by 17AAGA

NSCLC cells differed widely in their ability to secrete VEGF into the culture media. Values ranged from approximately 1,000 pg/mL/24 h/10^6 cells (H460, H322) to as high as 15,000 pg/mL/24 h/10^6 cells (H358) (Fig 4A). Cobalt chloride ($CoCl_2$), which simulates hypoxic conditions by interfering with cellular oxygen sensing mechanisms [14], induced significant upregulation of VEGF secretion (Fig 4A). Forty-eight-hour exposure to 17AAGA (80 nmol/L) significantly suppressed basal VEGF secretion in cultured lung cancer cells, with the magnitude of inhibition ranging from 10% to 50%. Furthermore, 17AAGA suppressed hypoxia-induced upregulation of VEGF secretion; the magnitude of inhibition in treated cells ranged from 30% to 45% relative to untreated "hypoxic" cells. To confirm these findings, cells were incubated in a hypoxic chamber (95% N_2 and 5% O_2). As expected, profound augmentation of VEGF secretion was noted in response to hypoxia, which could be effectively inhibited by 17AAGA (either 80 or 160 nmol/L) in a dose-dependent manner in all cell lines examined (Fig 4B).

Inhibition of Matrigel Membrane Invasion

The ability of cancer cells to migrate through the artificial extracellular matrix membrane, Matrigel, frequently correlates with their invasiveness in vivo [16]. Forty-eight-hour exposure to 17AAGA (80 nmol/L) significantly inhibited the ability of H322 and H358 cells to migrate through Matrigel. This inhibitory effect of 17AAGA was profound in H322 cells, with up to a 60% reduction in the number of cells invading through the membrane (109 ± 28 cells/5 HPF of 17AAGA-treated cells vs 293 ± 42 cells/5 HPF in untreated control cells; $p < 0.001$) (Fig 5). 17AAGA inhibited the invasiveness of H358 cells by approximately 40% (116 ± 8 cells/5 HPF of 17AAGA-treated cells vs 190 ± 14 cells/5 HPF of control cells; $p < 0.01$).

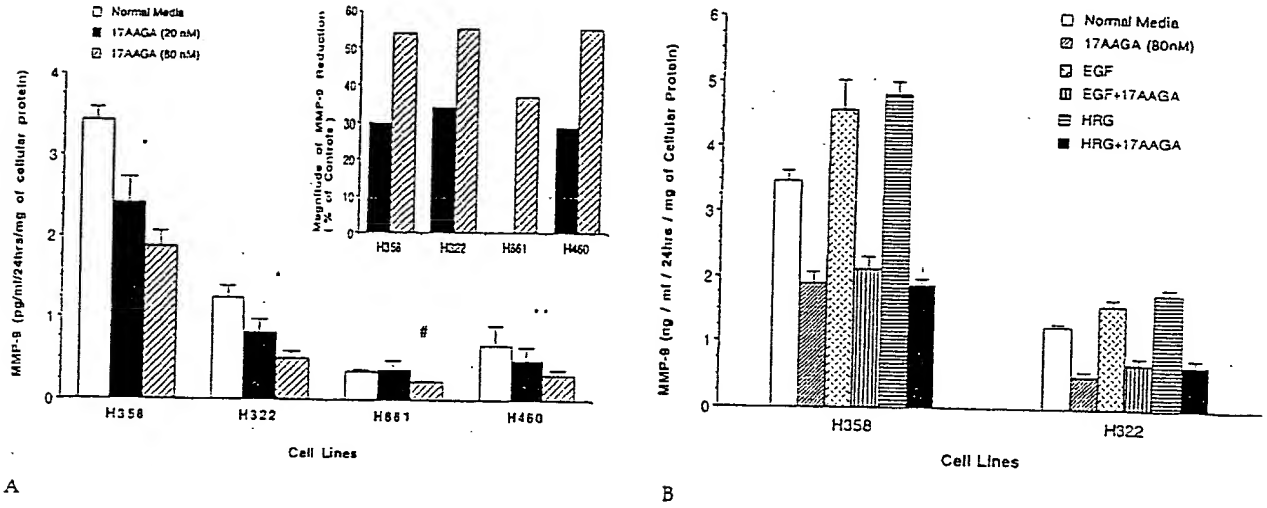

*Fig 3. (A) ELISA analysis of MMP-9 secretion by NSCLC cells, grown in the absence or presence of 17AAGA. Exposure of NSCLC cells to 17AAGA resulted in a dose-dependent reduction of MMP-9 levels in the conditioned media (*p < 0.001 vs normal media, #p < 0.01 17AAGA [80 nmol/L] vs normal media, ¯p < 0.05 17AAGA [80 nmol/L] vs normal media). The magnitude of inhibition of MMP-9 production is displayed in the upper right graph. Results are mean ± SD of four independent experiments. (B) Suppression of growth factor-mediated upregulation of MMP-9 secretion by 17AAGA (80 nmol/L) in H322 and H358 cells. Incubating H322 or H358 cells with 20 ng/mL of either epidermal growth factor (EGF) or heregulin α (HRG) for 48 hours induced 25% to 40% upregulation of MMP-9 secretion, which was effectively inhibited by 17AAGA. Results are means ± SD of three independent experiments.*

Comment

Better appreciation of the genetic and epigenetic factors that govern malignant transformation and metastasis may facilitate the development of more specific anticancer therapies. In addition to the development of drugs that specifically target the cell cycle machinery, considerable efforts have been focused on the evaluation of novel biologic or pharmacologic agents that suppress tumor growth or invasion by inhibiting neoangiogenesis or matrix metalloprotease activity. Activation of erbB protooncogenes profoundly disrupts cell cycle regulation [17], and enhances the expression of "pro-metastasis" phenotypes, namely decreased E-

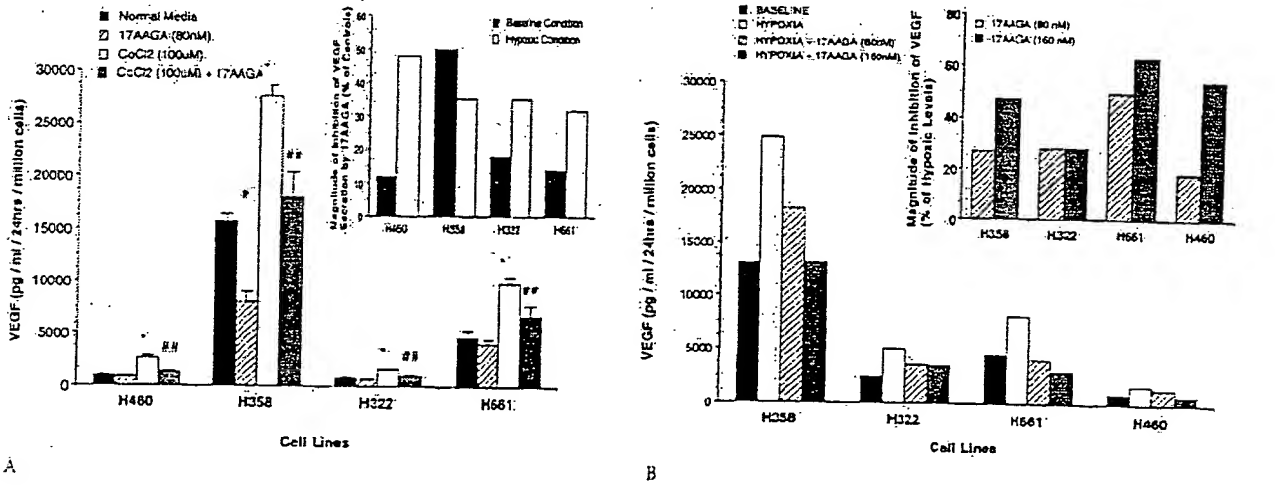

*Fig 4. (A) ELISA analysis of basal and hypoxia-stimulated VEGF secretion by 17AAGA. Significant upregulation of VEGF secretion into the culture supernatants by $CoCl_2$-simulated hypoxic condition was observed in all cell lines (*p < 0.001). 17AAGA at 80 nmol/L substantially suppressed the baseline (in H358 cells) and hypoxia-induced VEGF (in all four cell lines) secretion (*p < 0.001 vs baseline controls and #p < 0.001 versus hypoxia controls). The magnitude of inhibition of VEGF secretion by 17AAGA (expressed as percentages of baseline or hypoxia controls) is displayed in the upper right graph. Data are mean ± SD of three independent experiments. (B) ELISA analysis of VEGF levels in culture supernatants after physical hypoxia (< 5% O_2) with or without 17AAGA (80 or 160 nmol/L). The magnitude of 17AAGA-mediated inhibition of VEGF secretion (expressed as percentages of hypoxia controls) is displayed in the upper right graph. Data from a representative experiment are shown in this graph.*

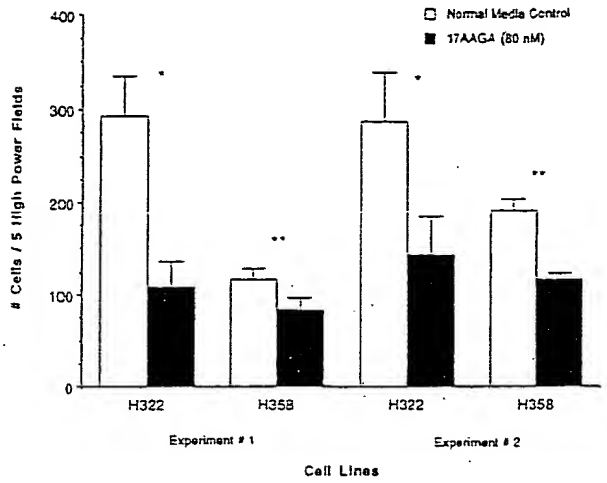

Fig 5. Inhibition of H322 and H358 cell invasion through Matrigel. Exposure of cells to 80 nmol/L 17AAGA for 48 hours reduced their invasion potential by 25% to 50% of that of normal cells. Data are means ± SD; each independent experiment was done in quadruplicate ($p < 0.005$, **$p < 0.05$).*

cadherin expression, increased VEGF and MMP secretion, as well as increased invasion through Matrigel [8–10]. These experimental results correlate closely with observations regarding c-erbB overexpression and aggressive clinical behavior of solid tumors [4–7]. These data provide the rationale for the development of treatment strategies specifically designed to abrogate expression or function of the c-erbB signal transduction pathways. Antagonistic anti-erbB1 and anti-erbB2 monoclonal antibodies, although not always effectively inhibiting tumor cell proliferation, have been shown to significantly upregulate E-cadherin expression, diminish VEGF and MMPs secretion, and reduce chemotaxis through extracellular matrix in vitro [10, 11]. Whereas these monoclonal antibodies may have limited clinical use from technical issues pertaining to delivery of macromolecules to tumor cells, low-molecular weight compounds such as 17AAGA that specifically inhibit erbB-mediated signal transduction pathways at nanomolar concentrations are of particular relevance.

In the present study, we sought to investigate the ability of 17AAGA to modulate expression of E-cadherin, MMP-9, and VEGF, each of which has been implicated in regulation of metastatic potential in tumor cells. The initial step of invasion and metastasis is the dissociation of cancer cells from the primary tumor mass resulting from aberrant expression of a variety of cell surface adhesion molecules, including integrin, immunoglobulins, secretins, and cadherins; the cadherins appear to be critical regulators of this process [18]. Cadherins are calcium-dependent transmembrane glycoproteins that mediate homophilic adhesion between cells. E-cadherin plays an important role in maintaining integrity of epithelial tissue; decreased E-cadherin expression or function correlates with tumor dedifferentiation, increased invasiveness, and lymph node metastases in a number of human carcinomas including lung, breast, esophagus, and prostate [16, 18]. E-cadherin has been referred to as an "antimetastasis" adhesion molecule, because the high propensity for metastasis formation in E-cadherin-negative cells can be reversed by restoration of expression of this adhesion molecule [19]. Previous studies have indicated an inverse relationship between erbB1 activation and E-cadherin expression in squamous cell cancers of skin and esophagus, as well as breast carcinomas [11, 20]. Upregulation of E-cadherin expression and inhibition of chemotaxis has been observed in H322 cells after exposure to an antagonistic monoclonal antibody directed against the erbB1 receptor [11].

MMPs are enzymes secreted by normal as well as cancer cells that degrade extracellular matrix and influence cell motility, tissue implantation, and angiogenesis. Considerable evidence indicates that MMP expression is increased in cancers relative to adjacent normal tissue, and levels of MMP expression correlate with tumor invasiveness and distant metastases [21]. There exist at least 10 isoforms of these proteases, each of which has unique substrate specificity. Secretion of MMPs is regulated by growth factors, some of which activate erbB1 or erbB2 mitogenic pathways. Inhibition of MMP activity either by recombinant tissue inhibitor of MMP or pharmacologic agents such as batimastat or marimastat decreases tumor growth, invasion, and metastasis in vivo [21]. More interestingly, reduction of MMP levels or inhibition of MMP activity has also been shown to suppress angiogenesis in vitro and in vivo [21].

Tumors smaller than 1 to 2 mm in diameter can receive nutrients by diffusion, but continued growth of the lesions is predicated on neoangiogenesis, which is regulated by complex mechanisms mediated by a balance between proangiogenic and antiangiogenic cytokines present simultaneously in the tumor and normal stromal tissues [22]. VEGF is one of the most potent proangiogenic cytokines, and secretion of VEGF by tumor cells may be sufficient to ensure neovascularization. The extent of neovascularization correlates with aggressive chemical behavior in a variety of malignancies including breast or prostate cancers and melanomas; vascular density and VEGF levels in tumor tissue are significant, independent predictors of systemic recurrence and diminished survival in patients with stage I NSCLC.

Although tumor dissemination, itself, does not require angiogenesis, interruption of angiogenesis should prevent the growth of metastatic deposits [22]. This is the basis of antiangiogenesis therapies using monoclonal antibodies against circulating VEGF or VEGF receptors on endothelial cells. Another strategy to inhibit angiogenesis involves targeting oncogene or tumor suppressor gene mutations directly influencing VEGF transcription through the use of Herceptin in breast cancer patients whose tumors overexpress erbB2, or potentially, by restoration of p53 expression using gene therapy techniques [10, 23]. Our approach

has been to use a pharmacologic agent to achieve similar endpoints while avoiding monoclonal antibodies or cumbersome, inefficient adenoviral vectors that may have limited use in clinical settings. Conceivably, the in vivo antiangiogenesis effect of 17AAGA may be more pronounced than anticipated on the basis of our in vitro data because this compound suppresses VEGF expression as well as MMP-9, another potent mediator of neoangiogenesis.

Migration through Matrigel depends on a cell's ability to detach from a multicellular aggregate, degrade extracellular matrix, and undergo chemotaxis. The relative contribution of each of these phenotypes to cell invasiveness is not known. 17AAGA-mediated inhibition of cell invasion through Matrigel is the most concrete in vitro evidence of the antimetastatic activity of this compound. The mechanisms responsible for 17AAGA-mediated upregulation of E-cadherin, as well as inhibition of tumor-derived VEGF and MMP-9 secretion, most likely depend on the depletion of erbB2 or erbB1 proteins and subsequent decreased mitogenic stimulation via erbB pathways. This notion is supported by the fact that these effects have been observed in cancer cells after treatment with antagonistic monoclonal antibodies against erbB1 and erbB2 receptors [10, 11]. However, additional mechanisms unrelated to inhibition of erbB signal transduction may be relevant given the effects of 17AAGA on a variety of other cell cycle regulatory proteins, including Raf-1 and cyclin-dependent kinase 4 [13].

In vivo toxicity profiles of geldanamycin and 17AAGA have been documented in small-animal models; renal and hepatic toxicities are dose-limiting events [24]. However, the nanomolar concentrations required to mediate the "antimetastasis" effects are well below the maximal tolerable doses reported in animal toxicity studies [24]. Underlying mechanisms notwithstanding, our findings extend those pertaining to the antiproliferative effects of 17AAGA. These current data, together with our previous observations concerning the ability of 17AAGA to sensitize NSCLC cells to paclitaxel [25], provide impetus for the evaluation of 17AAGA in lung cancer patients.

References

1. Walsh GL, O'Connor M, Willis KM, et al. Is follow-up of lung cancer patients after resection medically indicated and cost-effective? Ann Thorac Surg 1995;60:1563–70.
2. Logan DM, Lochrin CA, Darling G, et al. Adjuvant radiotherapy and chemotherapy for stage II or IIIA non-small-cell lung cancer after complete resection. Provincial Lung Cancer Disease Site Group. Cancer Prev Control 1997;1:366–78.
3. Meyer T, Hart IR. Mechanisms of tumour metastasis. Eur J Cancer 1998;34:214–21.
4. Veale D, Kerr N, Gibson GJ, Kelly PJ, Harris AJ. The relationship of quantitative epidermal growth factor receptor expression in non-small cell lung cancer to long term survival. Br J Cancer 1993;162–5.
5. Volm M, Drings P, Wodrich W. Prognostic significance of the expression of c-fos, c-jun and c-erbB-1 oncogene products in human squamous cell lung carcinomas. J Cancer Res Clin Oncol 1993;119:507–10.
6. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
7. Hsieh CC, Chow KC, Fahn HJ, et al. Prognostic significance of HER-2/neu overexpression in stage I adenocarcinoma of lung. Ann Thorac Surg 1998;66:1159–63.
8. Xu F-J, Stack S, Boyer C, et al. Heregulin and agnostic anti-p-185c-erbB2 antibodies inhibit proliferation but increase invasiveness of breast cancer cells that overexpress p185c-erbB2: increased invasiveness may contribute to poor prognosis. Clin Cancer Res 1997;3:1629–34.
9. Yu D, Wang S-S, Dulski KM, et al. c-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. Cancer Res 1994;54:3260–6.
10. Petit AM, Rak J, Hung MC, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 1997;151:1523–30.
11. Al Moustafa AE, Yansouni C, Alaoui-Jamali MA, O'Connor-McCourt M. Up-regulation of E-cadherin by an anti-epidermal growth factor receptor monoclonal antibody in lung cancer cell lines. Clin Cancer Res 1999;5:681–6.
12. Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. Cancer Chemother Pharmacol 1995;36:305–15.
13. An WG, Schnur RC, Neckers L, Blagosklonny M. Depletion of p185/erbB-2, Raf-1, mutant p53 proteins by geldanamycin derivatives correlates with antiprolifeartive activity. Cancer Chemother Pharmacol 1997;40:60–4.
14. Goldberg MA, Schneider TJ. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J Biol Chem 1994;269:4355–9.
15. Albini A, Iwamoto Y, Kleinman HK, et al. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res 1987;47:3239–45.
16. Luo J, Lubaroff DM, Hendrix MJ. Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. Cancer Res 1999;59:3552–6.
17. Lukas J, Bartkova J, Bartek J. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. Mol Cell Biol 1996;16:17–25.
18. Shibanuma H, Hirano T, Tsuji K, et al. Influence of E-cadherin dysfunction upon local invasion and metastasis in non-small cell lung cancer. Lung Cancer 1998;22:85–95.
19. Saiki I. Cell adhesion molecules and cancer metastasis. Jpn J Pharmacol 1997;75:215–42.
20. Tamura S, Shiozaki H, Miyata M, et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br J Surg 1996;83:1608–14.
21. Yu AE, Hewitt RE, Connor EW, Stetler-Stevenson WG. Matrix metalloproteinases. Novel targets for directed cancer therapy. Drugs Aging 1997;11:229–44.
22. Fidler IJ. Modulation of the organ microenvironment for treatment of cancer metastasis. J Natl Cancer Inst 1995;87:1588–92.
23. Bouvet M, Ellis LM, Nishizaki M, et al. Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. Cancer Res 1998;58:2288–92.
24. Eiserman JL, Sentz DL, Zuhowski EG, Ramsland TS. Plasma pharmacokinetics and tissue distribution of 17-allylamino-geldanamycin (NSC330507), a prodrug for geldanamycin, in

CD2F1 mice and Fisher 344 rats [Abstract]. Proceedings of the American Association for Cancer Research 1997;38:308.
25. Nguyen DM, Chen A, Mixon A, Schrump DS. Sequence-dependent enhancement of paclitaxel toxicity in non-small cell lung cancer by 17-allylamino 17-demethoxygeldanamycin. J Thorac Cardiovasc Surg 1999;118:908–15.

DISCUSSION

DR ROBERT J. KEENAN (Pittsburgh, PA): One of the things that struck me was the effect or lack of effect on normal human bronchial epithelium. There were at least a couple of cell lines that seemed to have similar erbB-1 and erbB-2 expression to the normals, and yet there was no effect of your treatment on normal tissue. Can you comment on what the potential is for toxicity to normal tissues when there is expression of these genes in normals?

DR NGUYEN: Thank you. I am glad you brought up that issue. In the two graphs that I showed where we used normal human bronchial epithelial cells as a normal control, I did not show the effect of 17AAGA. First of all, I can say that when the normal human bronchial epithelial cells are grown for 96 hours in vitro to reach confluence to mimic the normal condition in vivo, in which there are very few mitotic activities, treating these cells with 17AAGA has no effect on their viability. Treating these cells with 17AAGA indeed does decrease the erbB-1 and erbB-2 expression, but that does not affect cell viability. So we know from our experiments that this drug is not toxic to normal human cells grown to confluence in vitro. I have not tested normal human cells in terms of their ability to secrete MMP-9 or VEGF at all.

DR KEENAN: Sometimes there is differential gene expression in the metastasis compared with the primary tumor. Do you have any data on whether or not some of the antimetastatic effect might be related to those differences in gene expression and whether your treatment might be more or less effective because of that?

DR NGUYEN: That is a very insightful question. No, I have not looked into all the phenotypes. That might contribute to it. The "prometastasis" phenotypes evaluated in this project are those that have been shown to associate with or actively play a role in the process of metastasis formation, but not the metastasis deposit itself.

DR JOHN R. BENFIELD (Los Angeles, CA): Could you tell us a bit more about the cell lines that you used? There is always the problem as to whether cultured cell lines for lung cancer, which, are hard to perpetuate, do in fact represent what happens in lung cancer in humans. What were the cell lines that you used?

DR NGUYEN: The H460 is a large-cell carcinoma, the H322 and H358 cells are bronchoalveolar carcinoma, and H661 is a large-cell carcinoma. So they are all non-small cell lung cancer of different subtypes.

DR BENFIELD: And they have been perpetuated for how many passages? Are these well-established cell lines?

DR NGUYEN: Yes, these cells are well-established, available from ATCC.

DR BENFIELD: Thank you. You alluded to the fact that you are ready now for in vivo testing of the agent: There are at least two ways in which you could do that preclinically. There are hamsters, non-small cell lung cancer models that are rather well established (Benfield JR, Malkinson AM, Schuller HM, Sunday ME. Animal models of lung cancer. In: Kane MA, Bunn PA Jr., eds. Biology of Lung Cancer. New York: Marcel Dekker, 1998: 247–93). The other thing that you might want to consider is placing some of the human tumors into nude mice, and then trying the agent while the nude mice are accepting the tumor.

DR NGUYEN: Thank you very much. I appreciate your suggestion.

To be specific about what we are doing, we create either a tumor mass in the flank or actually inject the H358 cells intravenously via lateral tail veins of nude mice to create a metastasis model and then treat the animals at a different time point after tumor cell inoculation with 17AAGA. So this is the experiment that we are carrying out right now.